(54) Title: TUMOUR MARKERS

(57) Abstract

A method of determining the immune response of a mammal to circulating tumour marker proteins is described in which a sample of bodily fluid, for example plasma or serum, is contacted with a panel of two or more distinct tumour marker antigens. The presence of complexes between the tumour marker antigens and any autoantibodies to the antigens present in the sample are detected and provide an indication of an immune response to a circulating tumour marker protein. The method is useful for the diagnosis of cancer, particularly for identifying new or recurrent cancer in an otherwise assymptomatic patient.

TUMOUR MARKERS

The invention relates to methods of detecting or quantitatively measuring the immune response of a mammal to circulating tumour markers or tumour markers expressed on the surface of tumour cells, also to tumour marker antigens for use in these methods, to kits for performing the methods and to the use of these methods in the detection of cancer, in monitoring the progress of cancer, in detecting recurrent disease in cancer patients who have previously undergone anti-cancer treatment and in predicting the response of a cancer patient to a particular course of treatment.

The development and progression of cancer in a patient is generally found to be associated with the presence of markers in the bodily fluid of the patient, these "tumour markers" reflecting different aspects of the biology of the cancer (see Fateh-Maghadam, A. & Steilber, P. (1993) Sensible use of tumour markers. Published by Verlag GMBH, ISBN 3-926725-07-9). Tumour markers are often found to be altered forms of the wild type proteins expressed by 'normal' cells, in which case the alteration may be a change in primary amino acid sequence, a change in secondary, tertiary or quaternary structure or a change in post-translational modification, for example, abnormal glycosylation. Alternatively, wild type proteins which are up-regulated or over-expressed in tumour cells, possibly as a result of gene amplification or abnormal transcriptional regulation, may also be tumour markers.

Established assays for tumour markers present in

bodily fluids tend to focus on the detection of tumour
markers which reflect tumour bulk and as such are of
value late in the disease process, for example, in the
diagnosis of metastatic disease. The most widely used

5   of these markers include carcinoembryonic antigen
(CEA) and the glycoprotein termed CA 15.3, both of
which have been useful mainly as indicators of
systemic disease burden and of relapse following
therapy (Molina, R., Zanon, G., Filella, X. *et al*. Use

10   of serial carcinoembryonic antigen and CA 15.3 assays
in detecting relapses in breast cancer patients.
(1995) *Breast Cancer Res Treat* **36**: 41-48) These
markers are of limited use earlier in the disease
progression, for example in the screening of

15   asymptomatic patients. Thus, in the search for tumour
markers present in bodily fluid that are of use
earlier in the disease process the present inventors
have sought to identify markers which do not depend on
tumour bulk *per se*.

20   Differences between a wild type protein expressed
by 'normal' cells and a corresponding tumour marker
protein may, in some instances, lead to the tumour
marker protein being recognised by an individual's
immune system as 'non-self' and thus eliciting an

25   immune response in that individual. This may be a
humoral (i.e B cell-mediated) immune response leading
to the production of autoantibodies immunologically
specific to the tumour marker protein. Autoantibodies
are naturally occurring antibodies directed to an

30.   antigen which an individual's immune system recognises
as foreign even though that antigen actually
originated in the individual. They may be present in
the circulation as circulating free autoantibodies or
in the form of circulating immune complexes consisting

- 3 -

of autoantibodies bound to their target tumour marker
protein.

As an alternative to the direct measurement or
detection of tumour marker protein in bodily fluids,
5   assays could be developed to measure the immune
response of the individual to the presence of tumour
marker protein in terms of autoantibody production.
Such assays would essentially constitute indirect
detection of the presence of tumour marker protein.
10   Because of the nature of the immune response, it is
likely that autoantibodies can be elicited by a very
small amount of circulating tumour marker protein and
indirect methods which rely on detecting the immune
response to tumour markers will consequently be more
15   sensitive than methods for the direct measurement of
tumour markers in bodily fluids. Assay methods based
on the detection of autoantibodies may therefore be of
particular value early in the disease process and
possibly also in relation to screening of asymptomatic
20   patients, for example to identify individuals "at
risk" of developing disease.

Tumour marker proteins observed to elicit serum
autoantibodies include a particular class of mutant
p53 protein, described in US Patent No. 5,652,115,
25   which can be defined by its ability to bind to the 70
kd heat shock protein (hsp70). p53 autoantibodies can
be detected in patients with a number of different
benign and malignant conditions (described in US
5,652,115) but are in each case present in only a
30   subset of patients. For example, one study utilizing
an ELISA assay for detection of autoantibodies
directed against the p53 protein in the serum of
breast cancer patients reported that p53
autoantibodies were produced by 26% of patients and

- 4 -

1.3% of control subjects (Mudenda, B., Green, J. A.,
Green, B. *et al*. The relationship between serum p53
autoantibodies and characteristics of human breast
cancer.(1994) *Br J Cancer* **69**: 4445-4449.). A second
tumour marker protein known to elicit serum
autoantibodies is the epithelial mucin MUC1 (Hinoda,
Y. *et al*. (1993) *Immunol Lett*. **35**: 163-168; Kotera,
Y. *et al*. (1994) *Cancer Res*. **54**: 2856-2860).

In most cancers resulting from a progressive
accumulation of genetic alterations, such as breast
cancer, the presence of tumour markers in bodily
fluids reflects the development and progression of
disease but no single marker on its own summates all
clinically important parameters. For example, the
characteristics of a marker useful for diagnosis of
cancer may be quite different from markers which
convey information about prognosis. Furthermore, in
each clinical situation (i.e. diagnosis or prognosis)
different markers may be required when dealing with
primary cancer and secondary (metastatic) cancer and a
different marker again may be required to provide a
method of measuring the effectiveness of a particular
course of treatment. Different clinical situations
therefore require different biological markers and, as
has been observed with p53, not all patients express
the same set of tumour marker proteins. It is
therefore difficult to envisage any one single tumour
marker being universally applicable to all patients in
all stages of disease.

It is an object of the present invention to
provide an improved assay system for the detection of
bodily fluids-borne tumour markers which is more
generally useful in all patients and in a variety of

- 5 -

different clinical situations.

Accordingly, in a first aspect the invention provides a method of detecting the immune response of a mammal to circulating tumour marker proteins or tumour cells expressing said tumour marker proteins, which method comprises steps of:

(a) contacting a sample of bodily fluids from said mammal with a panel of two or more distinct tumour marker antigens;

(b) determining the presence or absence of complexes of said tumour marker antigens bound to autoantibodies present in said sample of bodily fluids, said autoantibodies being immunologically specific to said tumour marker proteins.

whereby the presence of said complexes is indicative of the immune response to circulating tumour marker proteins or tumour cells expressing said tumour marker proteins.

The method of the invention, which may be hereinafter referred to as a 'panel assay', utilises a panel of two or more tumour marker antigens to monitor the overall immune response of an individual to a tumour or other carcinogenic/neoplastic change. The method thus provides essentially a 'profile' of the immune response for that individual, indicating which tumour markers elicit an immune response resulting in autoantibody production. The method of the invention is preferred for the detection of an immune response resulting in the production of circulating free autoantibodies.

- 6 -

Because the assay method of the invention
performed on a sample of bodily fluids taken from the
patient it is essentially non-invasive and can be
repeated as often as is thought necessary to build up
5   a profile of the patient's immune response throughout
the course of disease. As used herein the term
'bodily fluids' includes plasma, serum, whole blood,
urine, sweat, lymph, faeces, cerebrospinal fluid or
nipple aspirate. The type of bodily fluid used may
10   vary depending upon the type of cancer involved and
the use that the assay is being put to. In general,
it is preferred to perform the method on samples of
serum or plasma.

As will be illustrated in the Examples given
15   below, the use of a panel of two or more tumour marker
antigens to monitor autoantibody production is more
sensitive than the use of single markers and gives a
much lower frequency of false negative results.
The actual steps of detecting autoantibodies in a
20   sample of bodily fluids may be performed in accordance
with immunological assay techniques known *per se* in
the art. Examples of suitable techniques include
ELISA, radioimmunoassays and the like. In general
terms, such assays use an antigen which may be
25   immobilised on a solid support. A sample to be tested
is brought into contact with the antigen and if
autoantibodies specific to the tumour marker protein
are present in the sample they will immunologically
react with the antigen to form autoantibody-antigen
30   complexes which may then be detected or quantitatively
measured. Detection of autoantibody-antigen complexes
is preferably carried out using a secondary anti-human
immunoglobulin antibody, typically anti-IgG or anti-
human IgM, which recognise general features common to

- 7 -

all human IgGs or IgMs, respectively. The secondary
antibody is usually conjugated to an enzyme such as,
for example, horseradish peroxidase (HRP) so that
detection of autoantibody/antigen/secondary antibody
complexes is achieved by the addition of an enzyme
substrate and subsequent colorimetric,
chemiluminescent or fluorescent detection of the
enzymatic reaction products.

The panel assay of the invention uses a panel of
tumour marker-related antigens. The panel may be
tailored to detect a particular cancer, or a cancer at
a particular stage of development. The tumour marker
antigens may be wild type or mutant tumour marker
proteins isolated from samples of biological fluid
from normal individuals or from cancer patients or
from cell lines expressing the tumour marker protein
or they may be full length recombinant tumour marker
proteins, viral oncogenic forms of tumour marker
proteins or antigenic fragments of any of the
aforementioned proteins. The term 'antigenic
fragment' as used herein means a fragment which is
capable of eliciting an immune response.

The panel assay may be performed in a multi-well
format in which each one of the two or more antigens
is placed in a separate well of a multi-well assay
plate or, alternatively, in a single-pot format in
which the entire panel of antigens is placed in a
single container. The panel assay may be performed in
a qualitative format in which the objective is simply
detection of the presence or absence of autoantibodies
or in a quantitative format which provides a
quantitative measurement of the amount of
autoantibodies present in a sample.

Preferred markers for inclusion into the panel of

- 8 -

tumour marker antigens include the epidermal growth
factor receptor-related protein c-erbB2 (Dsouza, B. et
al. (1993) *Oncogene*. **8**: 1797-1806), the glycoprotein
MUC1 (Batra, S. K. et al. (1992) *Int. J.*
5   *Pancreatology*. **12**: 271-283) and the signal
transduction/cell cycle regulatory proteins Myc
(Blackwood, E. M. et al. (1994) *Molecular Biology of
the Cell* **5**: 597-609), p53 (Matlashewski, G. et al.
(1984) *EMBO J*. **3**: 3257-3262; Wolf, D. et al. (1985)
10   *Mol. Cell. Biol*. **5**: 1887-1893) and ras (or Ras)
(Capella, G. et al. (1991) *Environ Health
Perspectives*. **93**: 125-131), including the viral
oncogenic forms of ras which can be used as antigens
to detect anti-ras autoantibodies, and also BRCA1
15   (Scully, R. et al. (1997) *PNAS* **94**: 5605-10), BRCA2
(Sharan, S. K. et al. (1997) *Nature*. **386**: 804-810),
APC (Su, L. K. et al. (1993) *Cancer Res*. **53**: 2728-
2731; Munemitsu, S. et al. (1995) *PNAS* **92**: 3046-50),
CA125 (Nouwen, E. J. et al. (1990) *Differentiation*.
20   **45**: 192-8) and PSA (Rosenberg, R. S. et al. (1998)
Biochem Biophys Res Commun. **248**: 935-939). As
aforementioned, the assays can be formed using tumour
marker antigens which are forms of these proteins
isolated from human bodily fluids or from cultured
25   cells or antigenic fragments thereof or full length or
truncated recombinant proteins or antigenic fragments
thereof.

Preferably the tumour marker antigens are
labelled with biotin so that they can easily be
30   attached to a solid support, such as a multi-well
assay plate, by means of the biotin/avidin or
biotin/streptavidin interaction. Tumour marker
antigens labelled with biotin may be referred to

- 9 -

herein as 'biotinylated' proteins. To facilitate the
production of biotinylated tumour marker antigens for
use in the assay methods of the invention, cDNAs
encoding a full length recombinant tumour marker
5   protein, a truncated version thereof or an antigenic
fragment thereof may be expressed as a fusion protein
labelled with a protein or polypeptide tag to which
the biotin co-factor may be attached via an enzymatic
reaction. A useful system for the expression of
10   biotinylated fusion proteins is the PinPoint™ system
supplied by Promega Corporation, Madison WI, USA. The
present inventors have surprisingly found that with
the use of biotinylated tumour marker antigens as
antigens they are able to detect autoantibodies in a
15   much higher percentage of patients than is observed
using non-biotinylated antigen.

    The assay method of the invention may be employed
in a variety of different clinical situations such as,
for example, in the detection of primary or secondary
20   (metastatic) cancer, in screening for early neoplastic
or early carcinogenic change in asymptomatic patients
or identification of individuals 'at risk' of
developing cancer (particularly breast cancer, bladder
cancer, colorectal cancer or prostate cancer) in a
25   population or asymptomatic individuals, in the
detection of recurrent disease in a patient previously
diagnosed as carrying tumour cells who has undergone
treatment to reduce the number of tumour cells or in
predicting the response of an individual with cancer
30   to a course of anti-cancer treatment.

    The assay method of the invention is suitable for
detection of many different types of cancer, of which
examples are breast, bladder, colorectal, prostate and
ovarian. The assay of the invention may complement

existing methods of screening and surveillance. For
example in the case of primary breast cancer it could
be used to alert clinicians to biopsy small lesions on
mammograms which radiographically do not appear

5   suspicious or to carry out breast imaging or to repeat
imaging earlier than planned. In the clinic, the assay
method of the invention is expected to be more
objective and reproducible compared to current imaging
techniques (i.e. mammography and ultrasound), the

10   success of which can be operator-dependent.
As aforesaid the panel of tumour marker antigens
may be tailored having regard to the particular
application. A panel of at least p53 and c-erbB2 is
particularly useful for many types of cancer and can

15   optionally be supplemented with other markers having a
known association with the particular cancer, or a
stage of the particular cancer, to be detected. For
example for breast cancer the panel might include MUC
1 and /or c-myc and/or BRCA1 and/or BRCA2 and/or PSA

20   whereas bladder cancer the panel might optionally
include MUC 1 and/or c-myc, for colorectal cancer ras
and/or APC , for prostate cancer PSA and/or BRCA 1 or
for ovarian cancer BRCA1 and/or CA125. There are
other preferred embodiments in which p53 or c-erbB2

25   are not necessarily essential. For example, in the
case of breast cancer suitable panels could be
selected from the following:
p53 and MUC 1 with optional c-erbB2 and/or c-myc,
and/or BRCA1 and/or BRCA2 and/or PSA;

30   p53 and c-myc with optional c-erbB2 and/or MUC1
and/or BRCA1 and/or BRCA2 and/or PSA;
p53 and BRCA1 with optional c-erB2 and/or MUC 1
and/or c-myc and/or BRCA2 and/or PSA;
p53 and BRCA2 with optional c-erbB2 and/or MUC 1

and/or c-myc and/or BRCA1 and/or PSA;

c-erbB2 and MUC 1 with optional p53 and/or c-myc,
and/or BRCA1 and/or BRCA2 and/or PSA;

c-erbB2 and c-myc with optional p53 and/or MUC1
5   and/or BRCA1 and/or BRCA2 and/or PSA;

c-erbB2 and BRCA1 with optional p53 and/or MUC 1
and/or c-myc and/or BRCA2 and/or PSA;

c-erbB2 and BRCA2 with optional p53 and/or MUC 1
and/or c-myc and/or BRCA1 and/or PSA;

10

In the case of colorectal cancer suitable panels could
be selected from the following:

p53 and ras with optional c-erbB2 and/or APC;

p53 and APC with optional c-erbB2 and/or Ras;
15   Ras and APC with optional p53 and/or c-erbB2

In the case of prostate cancer suitable panels could
be selected from the following:

p53 and PSA with optional BRCA1 and/or c-erbB2;
20   c-erbB2 and PSA with optional p53 and/or BRCA1.

In the case of ovarian cancer suitable panels could be
selected from the following:

p53 and CA125 with optional c-erbB2 and/or BRCA1;
25   c-erbB2 and CA125 with optional p53 and/or BRCA1.

In a second aspect the invention provides a
method of determining the immune response of a patient
to two or more circulating tumour marker proteins or
30   to tumour cells expressing said tumour marker proteins
and identifying which one of said two or more tumour
marker proteins elicits the strongest immune response
in the patient, the method comprising contacting a
sample of bodily fluids from said patient with a panel

- 12 -

of two or more distinct tumour marker antigens,
measuring the amount of complexes formed by binding of
each of said tumour marker antigens to autoantibodies
present in the sample of bodily fluids, said
5   autoantibodies being immunologically specific to said
tumour marker proteins and using the measurement
obtained as an indicator of the relative strength of
the immune response to each tumour marker protein and
thereby identifying which one of said two or more
10   tumour marker proteins elicits the strongest immune
response in the patient.

     The assay described above, which may be
hereinafter referred to as a 'selection assay' is
useful in the selection of a course of vaccine
15   treatment wherein the single tumour marker protein
identified as eliciting the strongest immune response
or a combination of markers eliciting strong immune
response is/are used as the basis of an anti-cancer
vaccine treatment.

20   Preferred tumour marker antigens for use in the
selection assay are any of the tumour marker antigens
mentioned above and preferably the antigens are
labelled with biotin. The actual steps of detecting
autoantibodies in a sample of bodily fluids may be
25   performed in accordance with known immunological assay
techniques, as described above for the panel assay.

     The invention also provides methods for the
detection or quantitative measurement of the immune
response of a mammal to a circulating tumour marker
30   protein or tumour cells expressing the tumour marker
protein wherein the tumour marker protein is MUC1, c-
erbB2, Ras, c-myc, BRCA1, BRCA2, PSA, APC, CA125 or
p53, the method comprising the steps of contacting a
sample of bodily fluids from the mammal with the

tumour marker antigen and determining the presence or
absence of complexes of the tumour marker antigen
bound to autoantibodies immunologically specific to
the tumour marker protein or antigenic fragment
thereof, whereby the presence of said complexes is
indicative of the immune response to said circulating
tumour marker protein or tumour cells expressing the
tumour marker protein.

The assays described above, which may be
hereinafter referred to as 'single marker assays', use
a single type of tumour marker as antigen rather than
using a panel of two or more tumour markers. The
single marker assays may be used in any clinical
situation, for example, screening for early neoplastic
or carcinogenic change in asymptomatic patients,
identification of individuals 'at risk' of developing
cancer, early diagnosis and early detection of
recurrence in a patient previously diagnosed as
carrying tumour cells which patient has undergone
treatment to reduce the number of said tumour cells or
in predicting the response of a patient to a course of
anti-cancer treatment, including surgery,
radiotherapy, immune therapy, vaccination etc.

The single marker assays are particularly useful
in situations where the tumour marker eliciting the
strongest immune response in a given patient has been
previously identified, possibly using the selection
assay described above. For example, in a situation in
which an initial selection assay has been performed to
establish which tumour marker elicits the strongest
immune response in a given patient, subsequent follow-
up, detection of recurrence or monitoring of treatment
may be carried out using a single marker assay to only
detect or measure autoantibodies to that tumour marker

- 14 -

previously identified as eliciting a strong immune
response in that patient.

The actual steps of detecting autoantibodies in a
sample of bodily fluids may be performed in accordance
5   with known immunological assay techniques, as
described above for the panel assay. Preferably the
tumour marker protein used as antigen is labelled with
biotin so that it may be easily attached to a solid
support by means of the biotin/avidin or
10   biotin/streptavidin interaction.

In a further aspect, the present invention
provides a preparation comprising a human MUC1 protein
which MUC1 protein manifests all the antigenic
characteristics of a MUC1 protein obtainable from the
15   bodily fluids of a patient with advanced breast
cancer.

Preferably the MUC1 protein exhibits altered
affinity for the antibodies B55, C595, BC4W154, DF3,
B27.29, 115D8, 27.1, SM3, Ma552, HMPV and BC2 compared
20   to MUC1 protein isolated from normal human urine.
Most preferably the MUC1 protein is isolated from the
serum of one or more human patients with advanced
breast cancer. This can be accomplished using the
protocol given in the Examples listed herein.

25   As will be described in detail in Example 2, the
present inventors have found immunological differences
between MUC1 isolated from normal individuals and MUC1
isolated from patients with advanced breast cancer.
Possibly as a result of these differences, the
30   inventors have found that the MUC1 protein isolated
from serum of patients with advanced breast cancer
(hereinafter referred to as ABC MUC1) is more
sensitive when used as antigen in an assay to detect
autoantibodies specific to MUC1 than either MUC1

- 15 -

isolated from urine of normal individuals, synthetic
MUC1 or MUC1 isolated from a range of different
cultured cells. MUC1 isolated from the serum of
patients with advanced breast cancer is therefore
5   preferred for use as antigen in the panel assay method
and the single marker assay methods described herein.

MUC1 has recently attracted interest as a target
for immunotherapy of adenocarcinomas and several Phase
I clinical trials involving different MUC1 vaccine
10   substrates, adjuvants and carrier proteins have been
carried out (Goydos, J. S. et al. (1996) *J Surgical
Res.* **63**: 298-304; Xing, P. X. et al. (1995) *Int. J
Oncol.* **6**: 1283-1289; Reddish, M. A. et al. (1996)
Cancer Immunol. Immunother. **42**: 303-309; Graham, R. A.
15   et al. (1996) *Cancer Immunol. Immunother.* **42**: 71-80).
Methods for the detection of anti-MUC1 autoantibodies
using MUC1 isolated from the serum of patients with
advanced breast cancer as antigen will be of
particular use in monitoring the success of MUC1
20   vaccine therapy. In this case the aim of the assay
will be to detect anti-MUC1 <u>antibodies</u> produced in
response to the vaccine rather than autoantibodies
i.e. antibodies produced in response to an exogenous
antigen introduced into the body by vaccination.
25   Methods for the detection of autoantibodies directed
to other tumour markers would also be of use in
monitoring the success of vaccine therapy using the
relevant tumour marker. For example, following
vaccination with a p53 antigenic preparation, the
30   presence of anti-p53 antibodies could be monitored
using the assay based on the use of biotinylated p53
antigen described in the examples given below.
Moreover, the panel assay method could also be used in
monitoring the success of vaccine therapy, for

- 16 -

·· example, in a situation where an individual has been
vaccinated with an antigenic preparation designed to
elicit antibodies to two or more different tumour
markers.

5   In a still further aspect the invention provides
a method of detecting recurrent disease in a patient
previously diagnosed as carrying tumour cells, which
patient has undergone treatment to reduce the number
of said tumour cells, which method comprises steps of
10   contacting a sample of bodily fluids from the patient
with MUC1 protein or an antigenic fragment thereof,
determining the presence or absence of complexes of
said MUC1 protein or antigenic fragment thereof bound
to autoantibodies present in said sample of bodily
15   fluids, said autoantibodies being immunologically
specific to MUC1, whereby the presence of said
complexes indicates the presence of recurrent disease
in said patient.

        The method described above may be repeated on a
20   number of occasions to provide continued monitoring
for recurrence of disease. The method is particularly
preferred for the monitoring of patients previously
diagnosed with primary breast cancer, colorectal
cancer, prostate cancer or bladder cancer, which
25   patients have undergone treatment (e.g. surgery) to
remove or reduce the size of their tumour. In this
instance, the presence of anti-MUC1 autoantibodies in
the patient's serum after treatment may be indicative
of recurrence of disease.

30   Also provided by the invention are assay kits
suitable for performing the methods for the detection
of autoantibodies described herein. Such kits include,
at least, samples of the tumour marker antigens to be
used as antigen in the assay and means for contacting

- 17 -

the sample to be tested with a sample of the antigen.

The contents of all documents, articles and
references cited herein are incorporated herein by
reference.

5   The present invention will be further understood
with reference to the following Examples and the
accompanying Figures in which:

Figure 1: shows the results of assays for
10   autoantibodies to MUC1, p53 and c-erbB2 in samples of
serum taken from 21 patients diagnosed with primary
breast cancer. Panel A: anti-p53 autoantibodies; Panel
B: anti-c-erbB2 autoantibodies and Panel C: anti-MUC1
autoantibodies. In each case, the dotted line
15   represents the cut-off value for normality.

Figure 2: shows reactivity profiles of MUC1 protein
isolated from normal human urine (panel A), ABC MUC1
isolated from the serum of patients with advanced
20   breast cancer (panel B) or MUC1 isolated from the
human breast cancer cell line ZR75-1 (panel C) with
various monoclonal anti-MUC1 antibodies.

Figure 3: shows continuous monitoring for recurrent
25   disease in three post-operative breast cancer
patients. Quantitative assays for anti-MUC1, anti-c-
erbB2 and anti-p53 autoantibodies and for the tumour
marker CA15-3 (TM) were performed on samples of serum
taken at two or three monthly intervals post-surgery.

30

Figure 4: shows the range of autoantibody levels found
in assays for autoantibodies to c-erbB2, c-myc, MUC1
and p53 in normal individuals and patients with early
primary breast cancer (PBC).

- 18 -

Figure 5: summarises the detection rate for primary
breast cancer in an analysis of autoantibody levels in
a series of healthy controls and patients with primary
breast cancer, PBC subdivided by Stage 1-i.e. lymph
5   node negative and Stage 2-i.e. lymph node positive and
patients with metastatic cancer at 100% confidence.

Figure 6: summarises the detection rate for primary
breast cancer in an analysis of autoantibody levels in
10   a series of healthy controls and patients with PBC
subdivided by Stage 1-i.e. lymph node negative and
Stage 2-i.e. lymph node positive and patients with
metastatic cancer at 95% confidence.

15   Figure 7: shows the sensitivity for primary breast
cancer in an analysis of autoantibody levels in a
series of healthy controls and patients with Stage 1
or Stage 2 primary breast cancer at 95% confidence.

20   Figure 8: shows the levels of autoantibodies to MUC1,
p53 and c-erbB2 in the serum of three patients
previously diagnosed with breast cancer measured
sequentially during follow-up until the patient
manifested recurrent disease.
25
Figure 9: shows the autoantibody levels in further
samples from the second patient in Figure 10 (REC at
36 months) taken up to recurrence and during treatment
for recurrence. Sequential measurements of
30   established tumour markers reflecting tumour bulk
(e.g. CA15-3 and CEA) were within the normal range
throughout this period (data not shown).

Figure 10: shows follow-up autoantibody levels in

- 19 -

post-operative serum samples from two patients, one
who did not develop recurrent disease (no REC) and the
other who did (REC at 36 months).

5   Figure 11: summarises the detection rates in an
analysis of autoantibody levels (p53, MUC1, c-erbB2
and c-myc) in samples of serum taken from patients
with urologically benign disorders and various stages
of bladder cancer.

10   * indicates patients which were benign with respect to
urology (i.e. did not have a urological malignancy),
but six cases (all with positive autoantibody status)
had evidence of other malignancies.
** Other malignancies were:- lung cancer, skin cancer,

15   adenocarcinoma of unknown primary. Evidence of other
neoplasia consisted of:- pleural effusion, ovarian
cysts, colon polyps.

Figure 12: summarises the detection rate for
20   colorectal cancer in an analysis of autoantibody
levels in the serum of healthy controls, patients with
colonic polyps and patients with colorectal cancer at
100% confidence compared to a pre-defined group of
healthy controls.

25

Figure 13: summarises the detection rate for
colorectal cancer in an analysis of autoantibody
levels serum of healthy controls, patients with
colonic polyps and patients with colorectal cancer at

30   at 95% confidence compared to a pre-defined group of
healthy controls.

Figure 14: summarises the detection rate in an
analysis of autoantibody levels in the serum of

- 20 -

healthy controls, patients with primary breast cancer
and asymptomatic women known to be BRCA1 mutant
carriers at 100% confidence compared to a pre-defined
group of healthy controls.

5

Figure 15: summarises the detection rate for prostate
cancer in an analysis of autoantibody levels in the
serum of healthy controls and patients with prostate
cancer at 95% confidence compared to a pre-defined
10   group of healthy controls.

Examples

15

Example 1-Isolation of ABC MUC1 from advanced breast cancer patients.
Method
        ABC MUC1 was purified from pooled sera taken from
20   20 patients with advanced breast cancer using
        immunoaffinity chromatography as follows:
        The mouse monoclonal anti-MUC1 antibody B55 (also
known as NCRC 11 and described by Ellis et al. (1984)
Histopathology. 8: 501-516 and in International patent
25   application No. WO 89/01153) was conjugated to CNBr-
sepharose beads. Pooled sera from patients diagnosed
with advanced breast cancer was diluted 1/10 in
phosphate buffered saline (PBS) and then incubated
with the antibody conjugated sepharose beads (25ml
30   diluted sera to 1ml packed volume of beads) overnight
at 4°C with rolling. The beads were then packed by
centrifugation and the supernatant removed. In order
to wash away unbound serum components the beads were
resuspended in PBS, rolled for 10 minutes, packed by

centrifugation and the supernatant removed. This
washing sequence was repeated 5 times (or until A280nm
of the supernatant was ~0). The washed beads were then
resuspended in 0.25M glycine pH 2.5, rolled at room

5   temperature for 10 minutes, packed by centrifugation
and the supernatant removed. This supernatant was
adjusted to pH 7 by the addition of Tris and stored at
4°C labelled 'glycine fraction'. The beads were then
resuspended in 1ml 25mM diethylamine (DEA) pH11,

10   rolled at room temperature for 10 minutes, packed by
centrifugation and the supernatant removed. This
supernatant was again adjusted to pH 7 by the addition
of Tris and stored at 4°C labelled '25 DEA fraction'.
The beads were finally resuspended in 1ml 100mM DEA

15   pH11, rolled at room temperature for 10 minutes,
packed by centrifugation and the supernatant removed.
The final supernatant was again adjusted to pH 7 by
the addition of Tris and stored at 4°C labelled '100
DEA fraction'. The MUC1 content of the three fractions

20   (glycine fraction, 25 DEA fraction and 100 DEA
fraction) was confirmed by ELISA using the mouse
monoclonal anti-MUC1 antibody C595 (commercially
available from Serotec).

25   Example 2-Immunological characterisation of ABC MUC1
isolated from the serum of patients with advanced
breast cancer.
       ABC MUC1 isolated from the serum at least 20
patients with advanced breast cancer according to the

30   procedure described in Example 1 can be distinguished
from MUC1 isolated from the urine of normal human
subjects (normal human urinary MUC1) on the basis of
altered affinity for the following mouse monoclonal
anti-MUC1 antibodies:

B55 (NCRC 11)

C595

BC4W154   Obtainable from Hybritech, Inc

DF3   Obtainable from Centocor

5   B27.29   Obtainable from Biomira, Inc

115D8   Obtainable from Centocor

27.1   Obtainable from Austin Research

            Institute

SM3   Obtainable from the Imperial Cancer

10   Research Fund

Ma552   Obtainable from CanAg

HMPV   Obtainable from Austin Research

            Institute

BC2   Obtainable from Austin Research

15   Institute

        Normal urinary MUC1 is available from Dr M.R.

    Price, Cancer Research Laboratories, The University of

    Nottingham, University Park, Nottingham. NG7 2RD,

20 United Kingdom.

        The affinity of each of the above antibodies for

    ABC MUC1, normal human urinary MUC1 and also MUC1

    protein purified from the human breast cancer cell

    line ZR75-1 (purified from a tissue culture

25 supernatant by gel filtration) was measured by

    performing colorimetric ELISA assays using each of the

    different antibodies and secondary anti-immunoglobulin

    antibodies conjugated to HRP. Following addition of

    the colorimetric substrate (TMB), measurements were

30 taken of OD at 650nm. The results of the ELISA assays

    are presented graphically in Figure 2. Values of Kd

    for the binding of several of these antibodies to ABC

    MUC1 and normal human urinary MUC1 are summarised in

    Table 1:

Table 1: Kd values for binding of monoclonal
antibodies to ABC MUC1 and normal human urinary MUC1.

Monoclonal	Kd vs ABC MUC1	Kd vs urinary MUC1
BC4W154	2.4×10^{-7}	1.7×10^{-9}
115D8	1×10^{-8}	3.38×10^{-8}
C595	2.4×10^{-8}	2.5×10^{-8}

Example 3-Cloning of biotinylated p53.

Method

Commercially available cDNA for p53 (*E. coli*
clone pBH53, deposited in the American Type Culture
Collection under accession number 79110) was cloned
into the PinPoint™ plasmid vector (Promega
Corporation, Madison WI, USA) using standard molecular
biology techniques. The PinPoint™ vector is designed
to facilitate the production of fusion proteins
comprising a biotinylation domain (consisting of a
fragment of a biotin carboxylase carrier protein)
fused N-terminally to the target protein of interest.
Care was therefore taken during the cloning procedure
to ensure that the reading frame of p53 was maintained
in the fusion protein. Procedures for cloning in
PinPoint™ vectors are described in detail in the
Promega Protocols and Applications Guide obtainable
from Promega Corporation, Madison WI, USA.

Fusion proteins expressed from the PinPoint ™
vector in *E. coli* are biotinylated by an enzyme system
of the *E. coli* host cells and may therefore be
purified or bound to an assay plate using conventional
avidin or streptavidin technology. For example,
procedures for purification of the fusion protein
using avidin covalently attached to a polymethacrylate
resin are described in the Promega Protocols and

Applications Guide obtainable from Promega
Corporation, Madison WI, USA.

Example 4-Cloning of c-erbB2.

5   Method

       Full-length cDNA encoding c-erbB2 was cloned from
the human breast cancer cell line ZR75-1, which can be
induced to up-regulate c-erbB2 expression by treatment
with the anti-cancer drug tamoxifen.

10   Two T25 flasks of sub-confluent ZR75-1 cells
(avaiable from the American Type Culture Collection
and from the European Collection of Cell Cultures,
deposit number ATCC CRL1500) grown in RPMI plus 10%
foetal calf serum were induced to express c-erbB2 by 4

15   day stimulation with tamoxifen at 7.5µM (see Warri et
al. (1996) Eur. J. Cancer. 32A: 134-140). The cells
were then harvested using trypsin/EDTA and washed
three times with PBS.

       mRNA was extracted from the cell pellet using a

20   Dynabead mRNA purification kit according to the
manufacturer's recommended protocol. The mRNA was
then used as a template for first strand cDNA
synthesis using the Pharmacia Ready-to-go™ T primed
first strand cDNA synthesis kit. cDNA/mRNA was then

25   blunt end ligated into the EcoRV site of the PinPoint™
vector. The ligation products were then transformed
into Top 10 F E. coli cells (Invitrogen) following the
manufacturer's supplied protocol and the transformed
cells grown overnight on LB agar plates containing

30   ampicillin. Colonies of the transformed E. coli were
copied onto nitrocellulose filter and then grown for 2
hours on LB agar containing ampicillin and IPTG (1mM).
The colonies on the nitrocellulose filter were fixed
and lysed (15 minutes in the presence of chloroform

- 25 -

vapour followed by 18 hours in 100mM Tris/HCL pH 7.8;
150mM NaCl; 5mM MgC12; 1.5% BSA; 1µg/ml DNase 1;
40µg/ml lysozyme).

Screening for colonies expressing anti-c-erbB2
reactive protein was carried out as follows:

1. Wash nitrocellulose filter three times in TNT
    (10mM Tris/HCl pH 8; 150mM NaCl; 0.05% Tween 20)
    then block for 60 minutes in TNT + 5% dried milk
    protein.

2. Incubate nitrocellulose filter for 2 hours at
    room temperature with mouse anti-c-erbB2 antibody
    (Ab-3 from Oncogene Research Products,
    Calbiochem).

3. Wash the filter three times in TNT then incubate
    overnight at 4°C with anti-mouse HRP conjugate.

4. Wash filter three times in TNT, twice in TN (10mM
    Tris/HCl pH 8; 150mM NaCl) then visualise
    colonies expressing anti-c-erbB2 reactive protein
    using chloronaphthol (6mg chloronaphthol in TN +
    6µl 30% H_2O_2).

5. After development (approximately 20 minutes
    treatment with chloronaphthol as described in
    step 4) wash filter with water and allow to air
    dry.

Colonies identified as positive for c-erbB2
expression were picked and grown up overnight in
liquid culture of LB + ampicillin and small amounts of
plasmid DNA and protein were prepared from the culture

- 26 -

for analysis. Plasmids containing a c-erbB2 cDNA
insert were identified using restriction enzyme
digestion and PCR using a primer pair specific to the
published c-erbB2 cDNA sequence, described by Yazici,
5   H. et al.(1996) Cancer Lett. 107: 235-239. DNA
sequence analysis could then be used to confirm 1) the
presence of a c-erbB2 insert and 2) that the reading
frame of c-erbB2 is maintained in the resultant
biotinylated fusion protein. Protein samples prepared
10   from E. coli cultures carrying a plasmid with a c-
erbB2 insert were analysed by SDS-PAGE and western
blotting to ensure that the correct protein was being
expressed.

15

Example 5-Detection of the immune response of patients
with primary breast cancer using a panel assay.

Methods:
20   (A) Preparation of biotinylated antigen
       E. coli transformed with the appropriate
PinPoint™ plasmid expressing biotinylated antigen
were grown in a 5ml overnight culture (LB + amp +
biotin) and the overnight culture used to inoculate a
25   150ml culture. The 150ml culture was grown to OD 0.4-
0.6 then expression of the fusion protein was induced
by the addition of IPTG to a final concentration of
1mM and the induced culture incubated at 25°C. The
bacterial cells were harvested by centrifugation and
30   then lysed by gentle sonication in a Tris/EDTA buffer
containing the protease inhibitor PMSF. Cellular
debris was removed by centrifugation at ~50,000g and
the resultant particle-free supernatant assayed by
avidin ELISA to confirm the presence of biotinylated

- 27 -

protein.

(B) c-erbB2/p53 autoantibody assay method

1. Standard 96 well microtitre assay plates were
    coated with avidin, using 50µl of a 1µg/ml
    solution per well, and allowed to air dry
    overnight. The plates were then washed once with
    PBS/Tween to remove residual salt crystals,
    blocked for 60 minutes with a solution of 2%
    (w/v) PVP (polyvinylpyrolidone 360) in PBS and
    washed three times using PBS/Tween.

2. Particle free supernatant containing the
    appropriate biotinylated antigen (prepared as
    described in section (1) above) was plated out at
    50µl per avidin-coated well and then incubated
    for 60 minutes at room temperature with shaking
    to allow the biotin/avidin binding reaction to
    take place. The plates were then washed four
    times with PBS/Tween.

3. Serum samples to be tested for the presence of
    autoantibodies (diluted 1/50 and 1/100 in
    PBS)were plated out in triplicate (50µl per well)
    and then incubated for 60 minutes with shaking to
    allow formation of any autoantibody/antigen
    complexes. Plates were then washed four times
    with PBS/Tween to remove unbound serum
    components.

4. 50µl of HRP conjugated anti-human IgG/IgM
    antibody (obtained from Dako and used at a
    dilution recommended by the manufacturer) was
    added to each well and incubated for 60 minutes

at room temperature with shaking. The plates
were then washed again four times with PBS/Tween.

5.   50µl of TMB was added to each well and
      measurements of OD at 650nm for each well of the
      assay plate were taken kinetically over a period
      of 10 minutes.

For each antigen, control assays were performed
following the procedure described above but using a
sample of protein induced from *E. coli* transformed
with a control PinPoint™ vector containing an out-of-
frame cDNA instead of the particle free supernatant
containing biotinylated antigen. As it will be
apparent to persons skilled in the art, the above
methodology can be adapted for use in the detection of
autoantibodies of any specificity with use of an
appropriate biotinylated antigen.

(C) MUC1 autoantibody assay
1.   ABC MUC1 isolated from the serum of patients with
      advanced breast cancer according to the method of
      Example 1 (all three fractions pooled) was
      diluted appropriately in PBS, plated out on a 96
      well microtitre assay plate at 50µl per well and
      left to dry overnight. The plate was then washed
      once with PBS/Tween to remove residual salt
      crystals, blocked for 60 minutes using a solution
      of 2% (w/v) PVP in PBS and washed three times
      with PBS/Tween.

2.   Serum samples to be tested for the presence of
      autoantibodies (diluted 1/50 and 1/100 in PBS)
      were plated out in triplicate, adding 50µl per

well, and incubated for 60 minutes at room
temperature with shaking. The plate was then
washed four times with PBS/Tween.

5   3.   50µl of HRP conjugated anti-human IgG/IgM
           antibody (obtained from Dako and used at a
           dilution recommended by the manufacturer) was
           added to each well and incubated for 60 minutes
           at room temperature with shaking. The plates were
10   then washed again four times with PBS/Tween.

     4.   50µl of TMB was added to each well and
           measurements of OD at 650nm for each well of the
           assay plate were taken kinetically over a period
15   of 10 minutes.

Results
        Pre-operative blood samples taken from 21
patients diagnosed with primary breast cancer were
20   assayed for the presence of autoantibodies against
MUC1, p53 and c-erbB2. The results of these assays are
shown in Figure 1 and summarised in Table 2, on page
30.

25

30

Table 2

Sample	anti-p53	Prediction	anti-c-erbB2	Prediction	anti-MUC1	Prediction	Combined
1	+	cancer	–	normal	+	cancer	CANCER
2	+/–	?	+/–	?	+/–	?	cancer
3	+	cancer	+/–	?	+	cancer	CANCER
4	+	cancer	+	cancer	+	cancer	CANCER
5	+	cancer	+	cancer	+/–	?	CANCER
6	–	normal	+	cancer	+/–	?	cancer
7	+	cancer	+	cancer	+	cancer	CANCER
8	+/–	?	+	cancer	+/–	?	CANCER
9	+	cancer	+	cancer	+	cancer	CANCER
10	+	cancer	+	cancer	–	normal	CANCER
11	+/–	?	+	cancer	+	cancer	CANCER
12	–	normal	+	cancer	–	normal	cancer
13	+	cancer	+	normal	+	cancer	CANCER
14	+/–	?	+	cancer	+	cancer	CANCER
15	+	cancer	+	normal	+	cancer	CANCER
16	–	normal	+	normal	+/–	?	?
17	+/–	?	–	normal	+	cancer	cancer
18	+	cancer	+	cancer	+	cancer	CANCER
19	+	cancer	+	cancer	+	cancer	CANCER
20	+	cancer	–	normal	+	cancer	CANCER
21	+	cancer	+/–	?	–	normal	cancer

Figure 1 shows the results of the assays for
autoantibodies specific to MUC1, c-erbB2 and p53. For
each set of data the dotted line represents the cut-
off value for normality. For the purposes of this
5   study the normal control patients were women who
clinically and/or mammographically had no evidence of
breast cancer at the time of taking the serum sample.
In order to establish the cut-off value for normality,
control assays were performed on a total of 30 normal
10   patients. Values below the dotted line fall within the
normal control range and were scored as negative (-)
in Table 2 whereas values above the dotted line were
scored as positive (+). Values which were difficult to
score as negative or positive with a reasonable degree
15   of certainty were scored +/-. Patients scoring
positive in at least two of the assays were identified
as strongly positive for breast cancer (indicated
"CANCER" in Table 2) ; patients scoring positive in at
least one of the assays were identified as probable
20   for breast cancer (indicated "cancer" in Table 2).

The results presented illustrate the predictive
value of the three autoantibody assays both when used
individually and when used as a panel. The use of a
single assay to predict breast cancer gave
25   approximately 40% of the results as a false negatives.
However, by combining the results from all three
assays only one patient appeared as a false negative
(<5%), 71% of patients were scored as strongly
positive for breast cancer (i.e. positive in at least
30   two assays) and 23% of patients were scored as
probable for breast cancer (i.e. positive in at least
one assay). The results also show that a group of
patients which have all been diagnosed with primary
breast cancer have different serological profiles in

terms of the immune response to their cancer. Thus, no
single one of the three autoantibody assays would be
useful in all primary breast cancer patients.

5   Example 6-Cloning of a ras antigen.
        Method
                cDNA encoding a mutant oncogenic form of ras
        (designated K-*ras*) was cloned from the cell line KNRK
        (Rat kidney, Kirsten MSV transformed, see Aaronson,
10   S.A. and Weaver, C.A. (1971) J. Gen. Virol. 13: 245-
        252; ATCC accession number CRL 1569). mRNA was
        extracted from the cell pellet using a Dynabead mRNA
        purification kit according to the manufacturer's
        recommended protocol. cDNA synthesis, cloning into
15   the EcoRV site of the PinPoint™ vector and
        transformation of *E. coli* was carried out as described
        in Example 4. Clones expressing ras were then
        identified by expression screening using the anti-ras
        antibody F234-4.2 from Calbiochem.
20

        Example 7 Cloning of c-myc.
        Method
                cDNA encoding human c-myc was cloned from the
        breast cancer cell line T47-D (European Collection of
25   Animal Cell Cultures accession number 85102201). mRNA
        was extracted from the cell pellet using a Dynabead
        mRNA purification kit according to the manufacturer's
        recommended protocol. cDNA synthesis, cloning into
        the EcoRV site of the PinPoint™ vector and
30   transformation of *E. coli* was carried out as described
        in Example 4. Clones expressing c-myc were then
        identified by expression screening using the anti-c-
        myc antibody 4111.1 from Unilever.

Example 8 Assay for ras and c-myc autoantibodies.

Biotinylated c-myc and ras antigens were prepared
from *E. coli* transformed with the appropriate
PinPoint™ plasmid vector expressing biotinylated c-myc
5   or biotinylated ras, as described in Example (5), part
(A). The assays for c-myc and ras autoantibodies were
then performed according to the protocol described in
Example (5), part (B).

10   Example 9 Method of detecting recurrent disease in a
patient previously diagnosed as carrying tumour cells.

A group of nine patients previously diagnosed
with primary breast cancer were selected. Pre-
operative serum samples were taken from each of these
15   patients prior to surgery for the removal of the
primary breast cancer. Follow-up serum samples were
then taken postoperatively at 2 or 3 monthly intervals
and during the same period of time the patients were
assessed clinically for signs of recurrent disease.
20   None of the patients received any post-operative
therapy until recurrence was diagnosed clinically. The
preoperative and post-operative serum samples from
each of the patients were assayed for the presence of
autoantibodies to MUC1, c-erbB2 and p53, using the
25   assay methods described above under Example 5, and
also for the presence of the commonly used serum
tumour marker protein CA15-3. The results of these
assays are summarised in Table 3, on pages 35 and 36
and results for three of the nine patients are
30   presented graphically in Figure 3.
Clinical signs of recurrent disease were scored as
follows:

LN   recurrent disease in the lymph nodes

LR   local recurrence

METS   distant metastases present

5   <u>Results</u>

In each of the patients at least one class of
autoantibody was observed to remain above normal
level. This suggests continued presence of the tumour
marker (immunogen) and hence continued presence of
10   tumour. Serum levels of the tumour marker protein
CA15-3 were not found to be predictive of recurrent
disease.

Table 3

Patient	Sample date	CA 15-3	Anti-p53	Prediction	Anti c-erbB2	Prediction	Anti MUC1	Prediction	Predicted	Recurrence	Date of first recurrence	DFI (months)
0001	12/88	11	-			Cancer	+	Cancer	CANCER	-		
	03/87	12	-		+	Cancer	+	Cancer	CANCER	-		
	05/87	13	-		+	Cancer	+	Cancer	CANCER	-		
	08/87	22	+/-	?	+	Cancer	+.	Cancer	CANCER	-		
	11/87	56	+/-	?	+	Cancer	+	Cancer	CANCER	METS		
	12/87	79	+/-	?	+	Cancer	+	Cancer	CANCER	METS		11
0002	01/87	16	-		+	Cancer	+/-	?	Cancer	-		
	05/87	8	-		+	Cancer	+/-	?	Cancer	-		
	08/87	10	-		+	Cancer	+	Cancer	CANCER	-		
	11/87	12	+/-	?	+	Cancer	+	Cancer	CANCER	-		
	02/88	16	-		+	Cancer	+	Cancer	CANCER	-	February 1989	23
0003	02/87	10	-		+	Cancer	-		Cancer	-		
	05/87	7	+	Cancer	+	Cancer	-		CANCER	-		
	08/87	8	+	Cancer	+	Cancer	-		CANCER	-		
	11/87	12	+	Cancer	+	Cancer	-		CANCER	-		
	02/88	12	+	Cancer	+	Cancer	-		CANCER	-		
	05/88	11	-		+	Cancer	-		Cancer	-	December 1989	34
0004	02/87	8	+	Cancer	++	Cancer	-		CANCER	-		
	04/87		+	Cancer	+	Cancer	-		CANCER	-		
	06/87	4	+	Cancer	+	Cancer	-		CANCER	-		
	12/87	0.4	+	Cancer	++	Cancer	-		CANCER	-		
	03/88	7	++	Cancer	++	Cancer	-		CANCER	-	February 1993	71

Table 3 continued

Patient	Sample date	CA 15-3	Anti-p53	Prediction	Anti c-erbB2	Prediction	Anti MUC1	Prediction	Predicted	Recurrence	Date of first recurrence	DFI (months)
0005	03/87	16	+/-	?	+	Cancer	-		Cancer	-		
	06/87	13	+/-	?	+	Cancer	-		Cancer	-		
	09/87	14	+	Cancer	+	Cancer	+/-	?	CANCER	-		
	12/87	17	+/-	?	+	Cancer	+/-	?	CANCER	-		
	03/88	16								-		
	05/88									LN		.15
0006	05/87	12	-		+	Cancer	+	Cancer	CANCER	-		
	07/87	15	-		+	Cancer	+	Cancer	CANCER	-		
	09/87	9	+/-	?	+	Cancer	+/-	?	Cancer	LR		4
	11/87	12	-		+	Cancer	+/-	?	Cancer	-		
	03/88	15	-		+/-	?	-		?	-		
	05/88	13	-		+/-	?	-		?	-		
000&	06/87	26	+	Cancer	++	Cancer	-		CANCER	-		
	08/87	28	+	Cancer	+	Cancer	-		CANCER	-		
	10/87	42	+	Cancer	+	Cancer	-		CANCER	-		
	12/87	105	+	Cancer	++	Cancer	+	Cancer	CANCER	METS	December 1987	6
0008	06/87	48	+	Cancer	+	Cancer	+	Cancer	CANCER	-		
	08/87	30	+	Cancer	+	Cancer	+	Cancer	CANCER	-		
	10/87	17	+	Cancer	+	Cancer	+	Cancer	CANCER	-		
	01/88	14	+	Cancer	+	Cancer	+	Cancer	CANCER	-		
	05/88	22	+	Cancer	+	Cancer	+/-	?	CANCER	LR	May 1988	11
0009	05/87	17	-		+/-	?	-		?	-		
	08/87	17	-		+	Cancer	-		Cancer	-		
	11/87	18	-		+	Cancer	-		Cancer	LR		6
	01/88	31	-		+	Cancer	+/-	?	Cancer	METS		8

- 37 -

Example 10 Retrospective analysis of a well
characterised series of healthy controls and patients
with early breast cancer.

The above-described methods for detecting
autoantibodies to MUC1, p53, c-erbB2 and c-myc were
used to carry out a retrospective study on a large
number of early (stage 1 and 2) breast cancer sera as
well as a large number of control serum samples from
individuals with no evidence of malignancy (control
group). The serum samples from patients were all
taken within a 4 week pre-operative period. At the
same time, the serum samples were assayed for the
presence of circulating antigen (MUC1 and c-erbB2)
using conventional tumour marker kits (used normally
in advanced disease only). This would allow an
assessment of whether the autoantibody assays are more
sensitive than the conventional antigen assays. As
used herein, the terms early or primary breast cancer
means that the primary tumour has a diameter of less
than 5cm. Stage 1 early breast cancer is defined as
lymph node negative; Stage 2 early breast cancer is
defined as lymph node positive.

In total, pre-operative serum samples from 200
patients diagnosed with primary breast cancer and 100
normal control samples were assayed for autoantibodies
against MUC1, p53, c-erbB2 and c-myc. The results are
summarised in Tables 4-7 and Figures 4-7.

Figure 4 depicts the range of autoantibody levels
found for each assay in normal individuals and
patients with early breast cancer. It is apparent
that cancer patients have a considerably higher level
of circulating autoantibodies to these markers than do
normal individuals. Using the range for the normal
individuals it is possible to set a 'cut-off' above

- 38 -

which no normal values should lie. Therefore, samples
with autoantibody levels above this cut-off can be
deemed to be positive for cancer. Cut-off points
determined in this manner were used to score the
5   results of the retrospective study in early breast
cancer patients.

The results presented in Tables 4-7 and Figures
5-7 demonstrate the predictive value of the four
autoantibody assays both individually and when used in
10   combination as a panel of assays. Table 4 indicates
the increased sensitivity of combining the results of
a number of assays. By using one assay on its own,
less than 50% of cancers are detected, however the
power of detection increases as more assays are added
15   to the panel until the combination of all four assays
allows 82% of primary cancers to be detected. Figure
7 shows the percentage of samples which are positive
in 0 out of 4 assays up to 4 out of 4 assays. This
provides good evidence that the panel assay is more
20   powerful in the detection of cancer than any one
single marker assay since not all patients with cancer
have raised autoantibodies to all markers.

Tables 5-7 summarise the detection rates in stage
1, stage 2 and in early breast cancer (i.e. stage 1
25   and 2) for various combinations of autoantibody
assays. The use of a single autoantibody assay to
predict breast cancer gives approximately 60-70% of
the results as false negatives in the stage 1 group;
and 50-60% in stage 2. However, by combining the
30   results from all four assays, 76% of stage 1 and 89%
of stage 2 cancers were positive in one or more assay.
The overall detection rate for early breast cancer
(i.e. both stage 1 and stage 2 cancers) using this
system was 82%. In both stage 1 and stage 2 cancer,

- 39 -

assaying for autoantibodies to MUC1 appeared to add
predictive power to any combination of assays.

The results for this study were obtained using a
100% confidence limit, in other words for a result to
5   be deemed positive it had to fall above the cut-off
for readings in the normal range. This normal range
was previously evaluated from a large number of normal
individuals and then confirmed using the control group
of 100 normal individuals mentioned above. Therefore,
10   within the normal control group, none of the samples
were found to be positive, meaning that the
sensitivity of the panel of autoantibody assays was
100% for the detection of early breast cancer (Figure
5).

15   Figures 6 and 7 demonstrate the detection rates
which are achievable if specificity is reduced from a
100% confidence level (no false positives) to a 95%
confidence level, where some degree of false positive
detection is expected. In this case, the cut-off
20   point is defined as the mean value plus twice the
standard deviation of the normal sample range. Using
this cut-off point, approximately 5% of the normal
samples were determined to be positive for cancer
(i.e. false positives); whilst detection of primary
25   cancer increased to approximately 94% (i.e. 6% false
negatives). Again, the greatest percentage of the
sample group were positive in only 1 out of the 4
assays, however, the percentage of samples that were
positive in all 4 assays increased considerably.
30   Since the above study was carried out
retrospectively, clinical data was available regarding
the initial diagnosis as well as clinical data
regarding the post-operative outcome (i.e. follow-up
data). This allowed analysis of the prognostic value

- 40 -

of the data obtained from the autoantibody assays.
Table 8 shows the correlations between serum levels of
autoantibodies to MUC1, p53, c-erbB2 and c-myc and a
number of clinical factors. For instance, the
5   presence of autoantibodies to any of the 4 tumour
associated proteins (MUC1, p53, c-erbB2 or c-myc)
appears to correlate with the development of a
recurrence. In other words, those patients who had
autoantibodies were more likely to go on to develop a
10   recurrence of their disease. In the case of
autoantibodies to MUC1, c-myc and c-erbB2, this was
most likely to be distant metastases, only
autoantibodies to p53 were not associated with the
later development of distant metastases with any
15   statistical significance. In fact, the presence of
autoantibodies to p53 was the weakest indicator of a
later recurrence of disease; furthermore, p53
autoantibodies correlated with disease free interval.
        Table 9 presents an analysis of whether the
20   degree of autoantibody positivity may be of value in
the prediction of which stage 1 tumour will go on to
develop a recurrence. At the present time, there is
little to indicate at the time of diagnosis whether a
patient with a stage 1 tumour (i.e. no evidence of
25   spread of tumour to the lymphatic system) will go on
to develop recurrent disease. As can be seen in Table
9, of those patients with stage 1 tumours from the
sample group that went on to develop recurrent
disease, 71% were positive in two or more autoantibody
30   assays. Of the patients with stage 1 tumours that
have not yet recurred, only 30% were positive in two
or more autoantibody assays.

Table 4: Sensitivity of autoantibody assays in the detection of early breast cancer.

	% PBC positive
Single marker assay	35-47
Two marker assay	51-60
Three marker assay	63-76
Four marker assay	82

Table 5: Sensitivity of autoantibody panel assays in the detection of stage 1 breast cancer.

	p53	c-erbB2	c-myc	MUC1
p53	38	48	58	59
c-erbB2		31	50	51
c-myc			41	55
MUC1				38
p53/c-erbB2			61	66
p53/c-myc				73
c-erbB2/c-myc				65
p53/c-erbB2/c-myc				76

Table 6: Sensitivity of autoantibody panel assays in
the detection of stage 2 breast cancer.

	p53	c-erbB2	c-myc	MUC1
p53	40	56	55	73
c-erbB2		42	56	73
c-myc			33	69
MUC1				56
p53/c-erbB2			65	84
p53/c-myc				80
c-erbB2/c-myc				84
p53/c-erbB2/c-myc				89

Table 7: Sensitivity of autoantibody panel assays in
the detection of primary breast cancer.

	p53	c-erbB2	c-myc	MUC1
p53	38	51	57	64
c-erbB2		35	53	59
c-myc			37	60
MUC1				47
p53/c-erbB2			63	73
p53/c-myc				76
c-erbB2/c-myc				72
p53/c-erbB2/c-myc				82

Table 8: Correlations between serum autoantibody level
and various clinical factors.

FACTOR	MUC1	p53	c-erbB2	c-myc
recurrence	✓	1✓4	✓	✓
local recurrence	1✓2	1✓2	1✓2	1✓4
distant metastases	✓	X	✓	✓
stage	X	X	X	X
grade	X	X	X	X
family history	X	X	X	X
disease free interval	X	✓	X	X
age	X	X	X	X
menopausal status	X	X	X	X

Key:

✓   Good correlation

1✓2   Moderate correlation

1✓4   Weak correlation

X   No correlation

Table 9: Analysis of the degree of positivity in
autoantibody assays for recurrent and non-recurrent
stage 1 breast cancer tumours.

	Negative-no autoantibodies detected	+ve auto-antibodies to one marker	+ve auto-antibodies to 2-4 markers
Recurrent	12%	17%	71%
Non-recurrent	22%	48%	30%

Example 11 Detection of autoantibodies in sequential
serum samples-application to the monitoring of disease
progression.

This study was carried out in order to assess
whether autoantibody assays could be useful in the

- 44 -

earlier detection of recurrent disease.

Levels of autoantibodies to MUC1, p53 and c-erbB2
in the serum of patients previously diagnosed with
breast cancer were measured sequentially during
5   follow-up until the patient manifested recurrent
disease. The results are summarised in Figures 8-10.
All three patients went on to develop recurrent
disease. In all three patients, autoantibody levels
were indicative of the presence of cancer. However,
10   there is no evidence from this group that autoantibody
levels decrease after removal of the primary tumour.
Figure 10 shows the levels of autoantibodies post-
operatively of a patient with non-recurrent disease
and a patient with recurrent disease. Autoantibody
15   levels in the patient with non-recurrent disease
remained below the cut-off point during the period of
sample collection (48 months). In the second patient,
whose disease recurred at 36 months, autoantibody
levels are seen to be steadily rising towards the cut-
20   off point, with c-erbB2 autoantibodies rising above
cut-off. Furthermore, as can be seen in Figure 9,
when further sequential samples are added to the
analysis, 3 out of the 4 assays become positive for
cancer and these levels then decrease again once
25   treatment of the recurrence is underway. This data
supports the utility of autoantibody assays in the
earlier detection of recurrent disease.

Example 12 Analysis of a series of patients with
30   bladder cancer and benign urological disorders.

Serum samples were collected from a group of 80
patients with bladder cancer/benign urological
disorders and analysed for the presence of
autoantibodies to MUC1, p53, c-erbB2 and c-myc using

- 45 -

the assay methods described above.

The data summarised in Table 10 shows that single assay sensitivities for bladder cancer detection range from 15-50% (as opposed to 35-47% for breast cancer).
5   The detection sensitivity using all 4 assays was 80%, similar to that found for early breast cancer.

Figure 11 shows the break down of detection rates between urologically benign disorders ('benign') and the three stages of bladder cancer. Upon further
10   investigation of the relevant clinical data it became apparent that 6 of the patients in the 'benign' group had evidence of other malignancies. These other malignancies were lung cancer, skin cancer and adenocarcinoma. Evidence of other malignancies were:
15   pleural effusion, ovarian cysts and colon polyps.
Serum samples from all 6 of these patients had been scored as positive for cancer using the panel of autoantibody assays, illustrating the general application of the panel assay to the detection of
20   cancers. Furthermore, it is known that some patients with stage PT1/2 and PT3/4 disease had previously received systemic therapy.

Table 10: Sensitivity of autoantibody assays in the
25   detection of bladder cancer.

	% positive
Single marker assay	15-50
Two marker assay	28-73
Three marker assay	46-76
Four marker assay	80

Table 11: Sensitivity of autoantibody panel assays in the detection of bladder cancer.

	p53	c-erbB2	c-myc	MUC1
p53	50	73	73	73
c-erbB2		17	28	36
c-myc			15	35
MUC1				24
p53/c-erbB2			76	76
p53/c-myc				75
c-erbB2/c-myc				46
p53/c-erbB2/c-myc				80

Example 13

Sensitivity of autoantibody assay in diagnosis of colorectal cancer.

An autoantibody assay as previously described was carried out on serum samples from patients with colorectal cancer using the tumour antigens c-myc, p53, c-erbB2 and K-ras individually and as a panel. The results are shown in figures 12 and 13. As has been demonstrated previously increased sensitivity is shown when a panel of antigens is used.

Example 14

Use of BRCA1 in panel assay for detection of breast cancer.

A BRCA1 antigen suitable for use in the detection of anti-BRCA1 autoantibodies was cloned from the breast cancer cell line MCF7 using an RT-PCR strategy. Briefly, mRNA isolated from MCF7 cells was reverse transcribed to give first-strand cDNA. These cDNA was used as a template for PCR using a primer pair

designed to amplify a product covering the first 1500
base pairs of the BRCA1 cDNA but including a known
mis-match mutation that leads to an early stop codon
and therefore the production of truncated protein.

5   Different sites for restriction enzyme digestion were
also incorporated into the forward and reverse PCR
primers to facilitate the cloning of the PCR product.
The PCR primers were as follows:

10   5'-GAC AGG ATC CGG ATG GAT TTA TCT GCT CTT CGC GTT G

5'-GCG GCC GCC CTC ATG TAG GTC TCC TTT TAC GC

     The PCR product obtained using these primers was
15   then cloned into the PinPoint™ vector and used to
transform *E. coli* Top 10 F cells, as described
hereinbefore. Clones expressing the fusion protein of
truncated BRCA1 antigen fused in-frame to the N-
terminal biotinylation domain were then identified by
20   expression screening, according to the procedure
described in Example 4, using the antibody MAB4132
from Chemicon.

     Biotinylated truncated BRCA1 antigen is then
prepared from *E. coli* transformed with the appropriate
25   PinPoint™ plasmid vector expressing the fusion
protein, as described in Example (5), part (A). The
assay for BRCA1 autoantibodies is then performed
according to the protocol described in Example (5),
part (B).

30   Figure 14 shows the results of a study in which
the above-described assays for autoantibodies to c-
myc, p53, c-erbB2, MUC1 and BRCA1 were performed
individually, as a panel and as a panel without BRCA1
to detect autoantibodies in samples of serum taken

- 48 -

from normal individuals, patients diagnosed with
primary breast cancer and BRCA1 mutation carriers. As
demonstrated previously, increased sensitivity is
shown when a panel of markers is used.

5

Example 15

Use of autoantibody panel assay for detecting prostate
cancer, incorporating PSA.

cDNA encoding human PSA was cloned from the cell
10 line T47-D using a protocol similar to that described
above for the cloning of c-erbB2. Briefly, the T47-D
cells were first stimulated with Apigenin at 10-5M as
described by Rosenberg et al. (1998) *Biochem Biophys
Res Commun.* **248**: 935-939. mRNA was then extracted and
15 cDNA synthesis, ligation into PinPoint™ and
transformation of *E. coli.* performed as described in
Example 4. Clones expressing PSA were identified
using an anti-PSA antibody. Biotinylated PSA antigen
was prepared from *E. coli* transformed with the
20 PinPoint™ vector expressing biotinylated PSA according
to the protocol described in Example (5), part (A).
The assay for PSA autoantibodies was then performed
according to the protocol described in Example (5),
part (B).

25 An autoantibody assay using the methods described
above was carried out on patients with prostate cancer
using c-myc, p53, c-erbB2, PSA and MUC 1 individually
and as a panel. The results are shown in figure 15
and confirm the increased sensitivity of such a panel
30 for detection of prostate cancer.

Example 15

Other tumour marker antigens.

CA125 can be affinity purified from the ovarian

cancer cell line OVRCAR-3 (available from the ATCC)
using Mab VK-8, as described by Lloyd, K. O. et al.
(1997) *Int. J. Cancer.* **71**: 842-850.

5   APC protein is expressed by the colorectal cancer
cell line SW480 (available from the ATCC) as described
by Munemitsu, S. et al. (1995) *PNAS* **92**: 3046-3050.

10

CLAIMS:

1.   A method of detecting the immune response of
a mammal to circulating tumour marker proteins or
tumour cells expressing said tumour marker proteins,
which method comprises steps of:

(a)   contacting a sample of bodily fluids from
said mammal with a panel of two or more
distinct tumour marker antigens;

(b)   determining the presence or absence of
complexes of said tumour marker antigens
bound to autoantibodies present in said
sample of bodily fluids, said autoantibodies
being immunologically specific to said
tumour marker proteins;

whereby the presence of said complexes is
indicative of the immune response to circulating
tumour marker proteins or tumour cells expressing
said tumour marker proteins.

2.   A method as claimed in claim 1 wherein at
least one of said two or more tumour marker antigens
is labelled with a protein or peptide tag.

3.   A method as claimed in claim 1 or claim 2
wherein at least one of said tumour marker antigens is
labelled with biotin.

4.   A method as claimed in any of claims 1 to 3
wherein at least one of said two or more tumour marker
antigens is selected from MUC1, p53, c-erbB2, Ras, c-

myc, BRCA1, BRCA2, PSA, APC or CA125 .

5.   A method as claimed in claim 4 wherein the
autoantibodies detected are indicative of cancer,
preferably breast, bladder, colorectal, prostate or
ovarian cancer.

6.   A method as claimed in claim 4 or claim 5
wherein said panel comprises at least p53 and c-erbB2.

7.   A method as claimed in claim 6 wherein the
cancer is bladder cancer and the panel also includes
MUC1 and/or c-myc.

8.   A method as claimed in claim 6 wherein the
cancer is colorectal cancer and the panel also
includes Ras and/or APC.

9.   A method as claimed in claim 6 wherein the
cancer is prostate cancer and the panel also includes
PSA and/or BRCA1.

10.   A method as claimed in claim 6 wherein the
cancer is breast cancer and the panel also includes
MUC1 and/or c-myc and/or BRCA1 and/or BRCA2 and/or PSA

11.   A method as claimed in claim 6 wherein the
cancer is ovarian cancer and the panel also includes
BRCA1 and/or CA125.

12.   A method as claimed in claim 5 wherein the
cancer is colorectal cancer and the panel is selected
from the following:
p53 and Ras optionally with c-erbB2 and/or APC,

·p53 and APC optionally with c-erbB2 and/or Ras, or
Ras and APC optionally with p53 and/or c-erbB2.

13.   A method as claimed in claim 5 wherein the
5   cancer is prostate cancer and the panel is selected
from the following:
p53 and PSA optionally with BRCA1 and/or c-erbB2, or
c-erbB2 and PSA optionally with p53 and/or BRCA1.

10   14.   A method as claimed in claim 5 wherein the
cancer is ovarian cancer and the panel is selected
from the following:
p53 and CA125 optionally with c-erbB2 and/or BRCA1, or
c-erbB2 and CA125 optionally with p53 and/or BRCA1.

·15

15.   A method as claimed in claim 5 wherein the
cancer is breast cancer and the panel is selected from
the following:
p53 and MUC 1 with optional c-erbB2 and/or c-myc,
20   and/or BRCA1 and/or BRCA2 and/or PSA,
p53 and c-myc with optional c-erbB2 and/or MUC1
and/or BRCA1 and/or BRCA2 and/or PSA,
p53 and BRCA1 with optional c-erB2 and/or MUC 1
and/or c-myc and/or BRCA2 and/or PSA,
25   p53 and BRCA2 with optional c-erbB2 and/or MUC 1
and/or c-myc and/or·BRCA1 and/or PSA,
c-erbB2 and MUC 1 with optional p53 and/or c-myc,
and/or BRCA1 and/or BRCA2 and/or PSA,
c-erbB2 and c-myc with optional p53 and/or MUC1
30   and/or BRCA1 and/or BRCA2 and/or PSA,
c-erbB2 and BRCA1 with optional p53 and/or MUC 1
and/or c-myc and/or BRCA2 and/or PSA, or
c-erbB2 and BRCA2 with optional p53 and/or MUC 1
and/or c-myc and/or BRCA1 and/or PSA.

16.   An assay kit suitable for performing the
assay method as claimed in any preceding claim, said
assay kit comprising a panel of two or more distinct
tumour marker antigens and means for contacting said
panel with a sample of bodily fluids.

17.   Use of the method of any of claims 1 to 15
in the detection of cancer, preferably breast,
bladder, colorectal, prostate or ovarian cancer.

18.   Use of the method of any of claims 1 to 15
in the detection of early neoplastic or early
carcinogenic change in asymptomatic patients.

19.   Use of the method of any of claims 1 to 15
in the detection of recurrent disease in a patient
previously diagnosed as carrying tumour cells, which
patient has undergone treatment to reduce the number
of said tumour cells.

20.   Use of the method of any of claims 1 to 15
in the identification of those individuals which are
at increased risk of developing cancer in a population
of asymptomatic individuals.

21.   The use as claimed in claim 20 in which
those individuals are at increased risk of developing
breast cancer, bladder cancer, prostate cancer,
colorectal cancer or ovarian cancer.

22.   Use of the method of any of claims 1 to 15
in determination of the tumour marker profile of an
individual suffering from cancer.

23.   The use as claimed in claim 19 wherein said
tumour marker profile is determined sequentially in
said individual as an indication of the course of
disease.

24.   Use of the method of any of claims 1 to 15
in predicting the response of an individual with
cancer to anti-cancer treatment.

25.   The use as claimed in claim 24 wherein said
anti-cancer treatment is hormone therapy,
chemotherapy, radiotherapy, anti-growth factor
therapy, immune therapy or vaccination.

26.   A method of determining the immune response
of a patient to two or more circulating tumour marker
proteins or to tumour cells expressing said tumour
marker proteins and identifying which one of said two
or more tumour marker proteins elicits the strongest
immune response in said patient, which method
comprises steps of:

(a)   contacting a sample of bodily fluids from
      said patient with a panel of two or more
      distinct tumour marker antigens;

(b)   measuring the amount of complexes formed by
      binding of each of said tumour marker
      antigens to autoantibodies present in said
      sample of bodily fluids, said autoantibodies
      being immunologically specific to said
      tumour marker proteins;

(c)   using the measurement obtained in part (b)

as an indicator of the relative strength of
the immune response to each tumour marker
protein and thereby identifying which one of
said two or more tumour marker proteins
5   elicits the strongest immune response in
said patient.

27.   A method as claimed in claim 23 wherein at
least one of said tumour marker antigens is labelled
10   with a protein or peptide tag.

28.   A method as claimed in claim 25 or claim 26
wherein at least one of said tumour marker antigens is
labelled with biotin.

15

29.   A method as claimed in any of claims 26 to
28 wherein at least one of said tumour marker antigens
is selected from MUC1, c-erbB2, c-myc, Ras, p53,
BRCA1, BRCA2, PSA, APC or CA125.

20

30.   Use of the method of any of claims 26 to 28
in the selection of a course of vaccine treatment.

31.   Use as claimed in claim 30 wherein one or
25   more tumour marker proteins identified as eliciting a
strong immune response in said patient is/are used as
the basis of a vaccine treatment.

32.   A preparation comprising a human MUC1
30   protein, wherein said MUC1 manifests all the antigenic
characteristics of a MUC1 protein obtainable from the
bodily fluids of a patient with advanced breast
cancer.

33. A preparation as claimed in claim 32, wherein the MUC1 protein exhibits altered affinity for the antibodies B55, C595, BC4W154, DF3, B27.29, 115D8, 27.1, SM3, Ma552, HMPV and BC2 compared to MUC1

5   protein isolated from normal human urine.

34. A preparation as claimed in claim 32 or claim 33 wherein the MUC1 protein has an affinity profile with the antibodies B55, C595, BC4W154, DF3,

10   B27.29, 115D8, 27.1, SM3, Ma552, HMPV and BC2 substantially identical to that shown in Figure 2.

35. A preparation as claimed in any one of claims 32 to 34 wherein the MUC1 protein is isolated

15   from the serum of one or more human patients with breast cancer, preferably advanced breast cancer or primary breast cancer.

36. A tumour marker protein substantially

20   equivalent to human MUC1 protein, which tumour marker protein is isolatable from the serum of one or more patients with advanced breast cancer and which tumour marker protein is characterised by exhibiting affinity for the antibody 115D8 3 to 6 times greater than the

25   affinity of MUC1 protein isolated from normal human urine for said antibody.

37. A method of detecting or quantitatively measuring autoantibodies immunologically specific to

30   MUC1 protein, which method comprises the steps of:

(a)   contacting a sample to be tested for said
         autoantibodies with the tumour marker
         protein of any one of claims 32 to 36;

  (b) determining the presence or absence of
     complexes of said tumour marker protein
     bound to said autoantibodies.

5   38. Use of the method of claim 37 in the
identification of those individuals which are at risk
of developing breast cancer or bladder cancer in a
population of asymptomatic individuals.

10   39. Use of the method of claim 37 in the
detection of cancer.

    40. Use of the method of claim 37 in the
detection of recurrent breast cancer in a patient
15 previously diagnosed as carrying breast cancer cells,
which patient has undergone treatment to reduce the
number of said breast cancer cells.

    41. Use of the method of claim 37 in the
20 detection of early neoplastic or early carcinogenic
change in asymptomatic patients.

    42. A method of quantifying the immune response
of a mammal to circulating tumour marker proteins or
25 tumour cells expressing said tumour marker proteins
wherein at least one of said tumour marker protein is
c-erbB2, Ras, c-myc, p53, BRCA1, BRCA2, APC, PSA or
CA125

30 which method comprises steps of:

  (a) contacting a sample of bodily fluids with at
     least one tumour marker antigen selected

from c-erbB2, ras, biotinylated c-myc,
BRCA1, BRCA2, APC, PSA, CA125 or
biotinylated p53;

5   (b)   measuring the quantity of complexes formed
              by binding of said at least one tumour
              marker antigen to autoantibodies present in
              said sample of bodily fluids, said
              autoantibodies being immunologically
10   specific to said tumour marker protein; and

        (c)   using the measurement obtained in (b) as a
              indicator of the amount of said
              autoantibodies present in said sample.
15

        43.   A method as claimed in claim 42 wherein said
tumour marker antigen is labelled with a protein or
peptide tag.

20   44.   A method as claimed in claim 42 or claim 43
wherein said tumour marker is labelled with biotin.

        45.   An assay kit suitable for performing the
method of any one of claims 42 to 44, said assay kit
25   comprising said tumour marker antigen, means for
contacting said antigen with said sample of bodily
fluids and means for facilitating quantitative
assessment of said complexes formed by binding of said
at least one tumour marker antigen to autoantibodies
30   present in said sample of bodily fluids.

        46.   Use of the method of any one of claims 42 to
44 in the detection of cancer.

47.   Use as claimed in claim 46 wherein the cancer is breast, colorectal, bladder, prostate or ovarian cancer.

48.   Use of the method of any one of claims 42 to 44 in the detection of early neoplastic or early carcinogenic change in asymptomatic patients.

49.   Use of the method of any one of claims 42 to 44 in the detection of recurrent disease in a patient previously diagnosed as carrying tumour cells, which patient has undergone treatment to reduce number of said tumour cells.

50.   Use of the method of any one of claims 42 to 44 in monitoring the progress of neoplastic disease.

51.   Use of the method of any one of claims 42 to 44 in the identification of those individuals which are at increased risk of developing cancer in a population of asymptomatic individuals.

52.   The use as claimed in claim 51 wherein those individuals are at increased risk of developing breast cancer, colorectal cancer, bladder cancer, prostate cancer or ovarian cancer.

53.   Use of the method of any one of claims 42 to 44 in predicting and/or measuring the response of an individual with cancer to anti-cancer treatment.

54.   The use as claimed in claim 53 wherein the anti-cancer treatment is hormone therapy, chemotherapy, radiotherapy, anti-growth factor

therapy, immune therapy or vaccination.

55.   A method of detecting the immune response of
a mammal to a circulating tumour marker protein or
5   tumour cells expressing said tumour marker protein
wherein said tumour marker protein is any one of the
tumour marker proteins listed in claim 4, said method
comprising the steps of:

10   (a)   contacting a sample of bodily fluids from
                   said mammal with MUC1, c-erbB2, Ras,
                   biotinylated c-myc, biotinylated p53, BRCA1,
                   BRCA2, APC, PSA or CA125 or an antigenic
                   fragment thereof;
15

            (b)   determining the presence or absence of
                   complexes of said tumour marker protein or
                   antigenic fragment thereof bound to
                   autoantibodies present in said sample of
20   bodily fluids, said autoantibodies being
                   immunologically specific to said tumour
                   marker protein or antigenic fragment
                   thereof;

25   whereby the presence of said complexes is
            indicative of the immune response to said
            circulating tumour marker protein or tumour
            cells, expressing said tumour marker protein.

30   56. A method as claimed in claim 55 wherein said
tumour marker antigen is labelled with a protein or
peptide tag.

            57.   A method as claimed in claim 55 or claim 66

- 61 -

wherein said tumour marker antigen is labelled with
biotin.

        58.   Use of the method of any one of claims 55 to
5   57 in the detection of cancer.

        59.   Use as claimed in claim 58 wherein the
cancer is breast, colorectal, bladder, prostate or
ovarian cancer.
10

        60.   Use of the method of any one of claims 55 to
57 in the detection of early neoplastic or early
carcinogenic change in asymptomatic patients.

15   61.   Use of the method of any one of claims 55 to
57 in the detection of recurrent disease in a patient
previously diagnosed as carrying tumour cells, which
patient has undergone treatment to reduce the number
of said tumour cells.
20

        62.   Use of the method of any one of claims 55 to
57 in monitoring the progress of cancer or other
neoplastic disease.

25   63.   Use of the method of any one of claims 55 to
57 in the identification of those individuals which
are at increased risk of developing cancer in a
population of asymptomatic individuals.

30   64.   The use as claimed in claim 63 wherein those
individuals are at increased risk of developing breast
cancer, colorectal cancer, bladder cancer, prostate
cancer or ovarian cancer.

- 62 -

65. Use of the method of any one of claims 55 to 57 in predicting and/or the response of an individual with cancer to anti-cancer treatment.
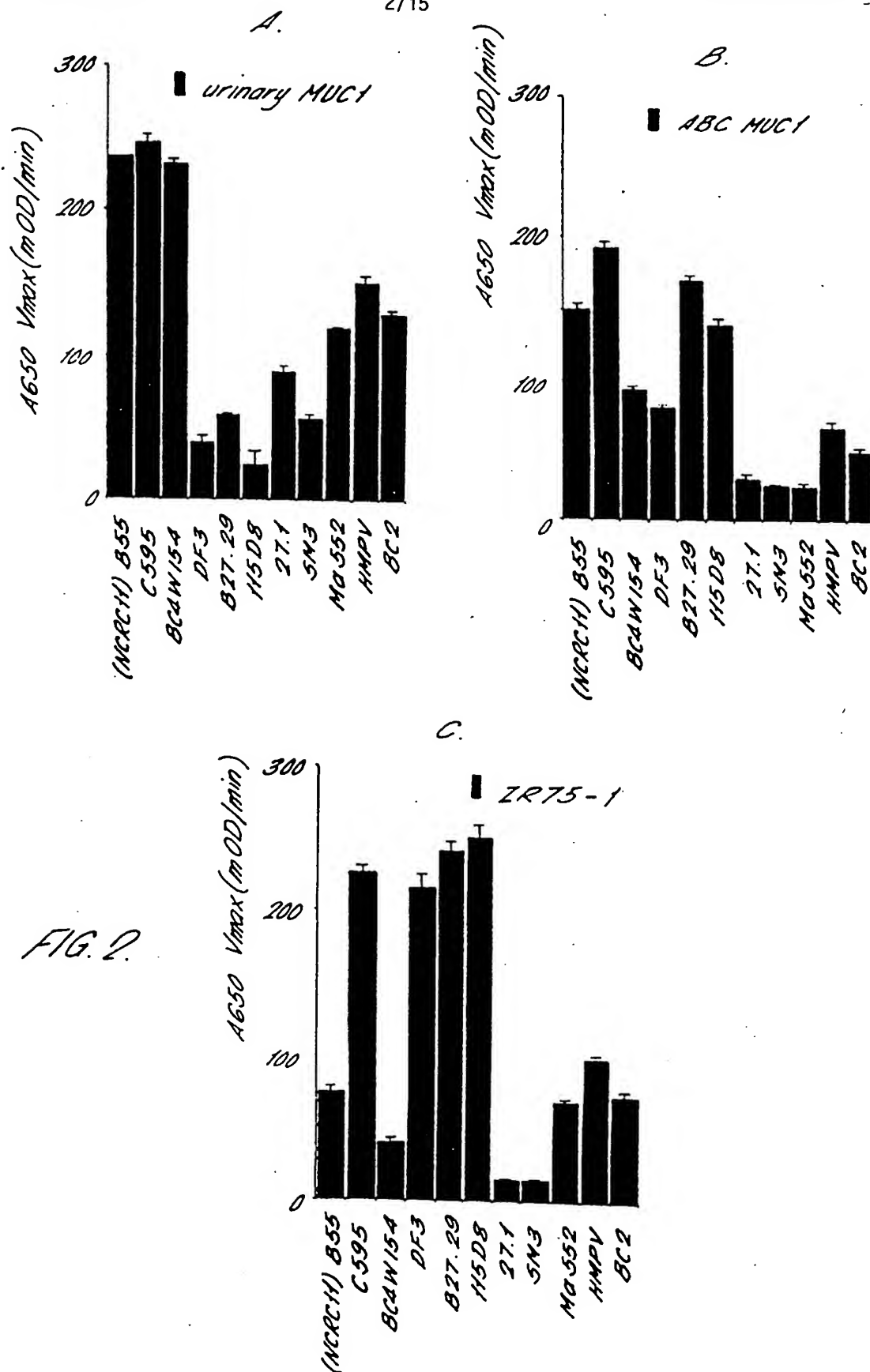
5   66. The use as claimed in claim 65 wherein the anti-cancer treatment is hormone therapy, chemotherapy, radiotherapy, anti-growth factor therapy, immune therapy or vaccination.

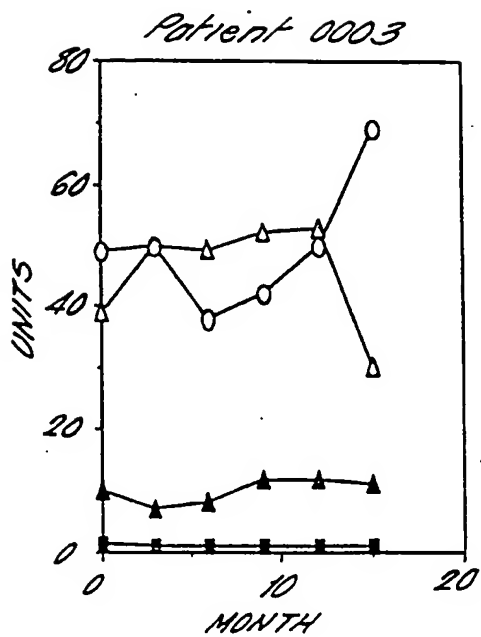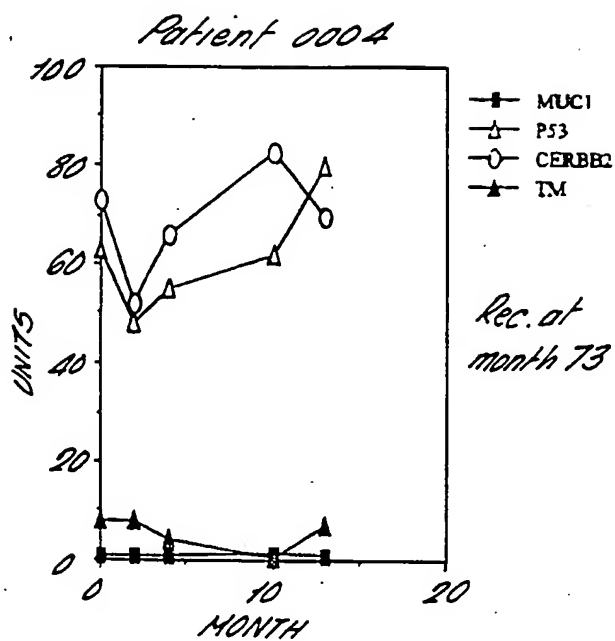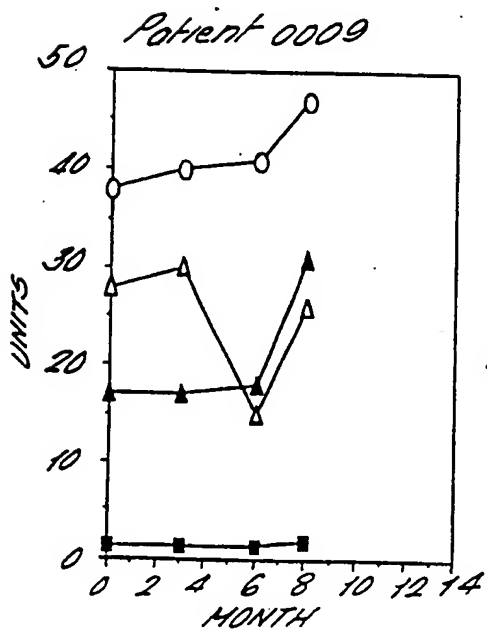10   67. A method of determining whether a vaccination procedure comprising challenging a patient with an immunogenic preparation comprising MUC1 protein or an antigenic fragment thereof has been successful in eliciting a humoral immune response,
15 which method comprises the steps of:

(a)   contacting a sample of serum from said patient with the modified MUC1 protein of any one of claims 32 to 36;

20

(b)   determining the presence or absence of complexes of said modified MUC1 protein bound to antibodies present in said sample of serum, said antibodies being
25   immunologically specific to MUC1 or an antigenic fragment thereof

whereby the presence of said complexes indicates that said vaccination procedure has been
30   successful in eliciting a humoral immune response.

68. A method of detecting recurrent disease in a patient previously diagnosed as carrying tumour cells,

which patient has undergone treatment to reduce the
number of said tumour cells, which method comprises
steps of:

5   (a)   contacting a sample of bodily fluids from
              said patient with MUC1 protein or an
              antigenic fragment thereof;

       (b)   determining the presence or absence of
10   complexes of said MUC1 protein or antigenic
              fragment thereof bound to autoantibodies
              present in said sample of sample of bodily
              fluids, said autoantibodies being
              immunologically specific to MUC1.
15

       whereby the presence of said complexes indicates
       the presence of recurrent disease in said
       patient.

20   69.   A method as claimed in claim 68 wherein said
       patient has been diagnosed with primary breast cancer
       prior to treatment to reduce the number of said tumour
       cells.

25   70.   Use of the method of claim 37 for selection
       of appropriate vaccine treatment.

       71.   Use of the method of claim 37 for predicting
       and/or measuring the response of an individual with
30   cancer to anti-cancer treatment.

       72.   The use as claimed in claim 71 wherein the
       anti-cancer treatment is hormone therapy,
       chemotherapy, radiotherapy, anti-growth factor

- . 64 -

therapy, immune therapy or vaccination

FIG. 1.

A.

B.

C.

FIG. 2.

FIG.3.

Patient 0003

Rec. at month 34

Patient 0004

Rec. at month 73

MUC1
P53
CERBB2
TM

Patient 0009
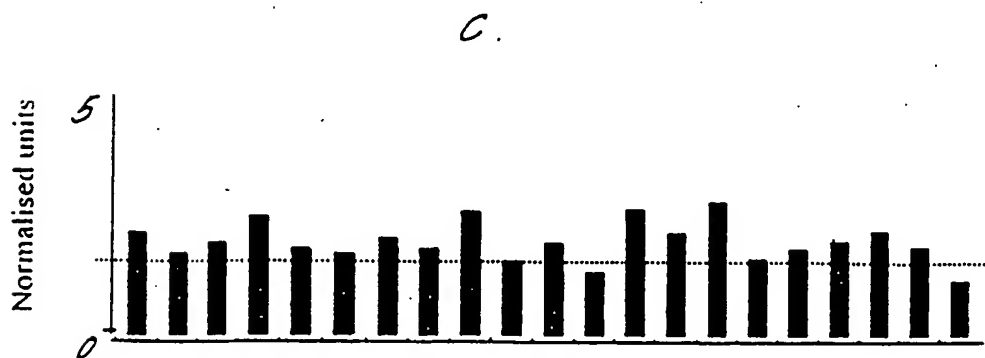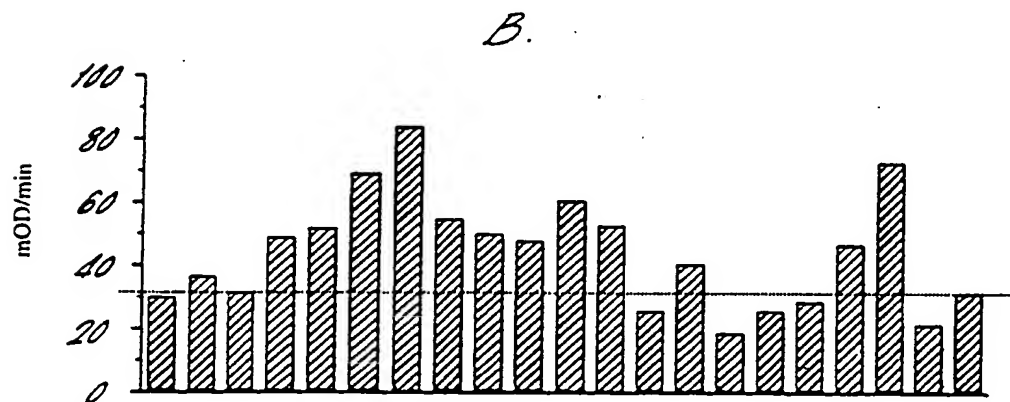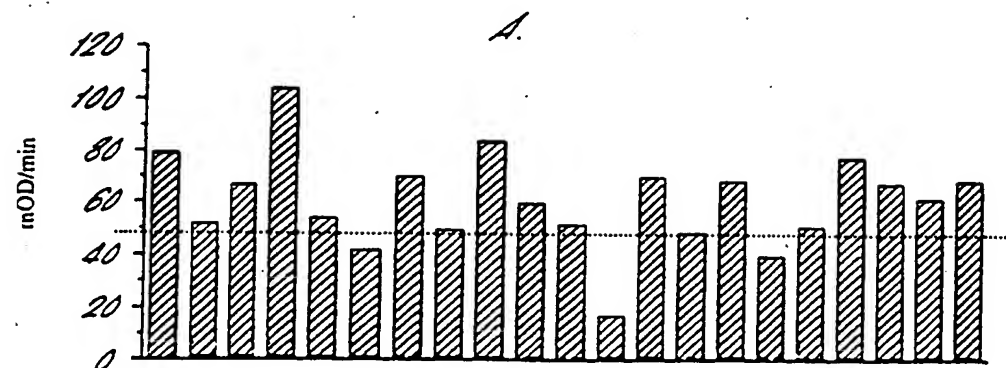
Rec. at month 8

FIG. 4

FIG.5

FIG. 6.

FIG. 7

FIG. 8.

FIG.9

p53
c-myc
c-erbB2
MUC1

Local recurrence

months post-op

FIG. 10

FIG. 11.

FIG. 12.

FIG. 13.

FIG. 14.

FIG. 15.